METABIOTIC GROWTH OF AND TOXIN FORMATION BY *CLOSTRIDIUM BOTULINUM*

ABSTRACT

Strains of Clostridium botulinum *types A, B and F were grown aerobically in metabiotic association with several aerobic and facultative microorganisms. In unbuffered media* Enterococcus faecalis *and* Pediococcus cerevisiae *produced enough acid to prevent toxin formation by* C. botulinum *but in buffered media toxin was formed.* Escherichia coli, Staphylococcus aureus, Bacillus subtilis, Yersinia enterocolitica, *strains of* Serratia, Pseudomonas, Alcaligenes *and 35 unidentified strains from mechanically deboned chicken homogenate produced conditions favorable for toxin production under aerobic conditions. The metabiotes did not inhibit spore formation nor did toxin production correlate with the appearance of spores.*

INTRODUCTION

Although *Clostridium botulinum* requires low oxygen tension for growth, it has been found capable of growing and producing toxin under "aerobic" conditions when other organisms are present. Early reports (Shippen 1919; Meyer 1929) indicated that *C. botulinum* would grow and produce toxin in the presence of yeasts, *Escherichia coli,* or *Staphylococcus aureus.* Francillon (1925) reported that *C. botulinum* would form toxin in the presence of *E. coli, S. aureus, Bacillus subtilis* and *Proteus* when cultivated under aerobic conditions. The studies of Quortrup and Holt (1941) and Quortrup and Sudheimer (1943) suggested that type C. botulism in wildfowl was caused by aerobic organisms depleting the oxygen in the lagoons where the birds fed. The early literature on this subject was reviewed by Ingram and Robinson (1951) and by Benjamin *et al.* (1956).

More recently Huhtanen *et al.* (1976) showed that *C. botulinum* would produce toxin in the presence of several common molds growing on tomato juice. A pH gradient was established during the growth of the molds which permitted the growth of *C. botulinum* under the mold mat but not at the bottom of containers where the acidity remained high. The implication was that the oxidation-reduction potential had also been lowered enough to allow growth of the organism.

Several recent outbreaks of botulism have been traced to foods generally considered not to be anaerobic enough for the growth of *C. botulinum*. MacDonald *et al.* (1985) reported on an outbreak of botulism associated with the consumption of sauteed onions; Solomon and Kautter (1986) subsequently reported that although several laboratory strains of *C. botulinum* type A produced small amounts of toxin in sauteed onions, the strain isolated from the outbreak produced high titers of toxin. Potato salad was implicated in outbreaks from Colorado [Centers for Disease Control (1978 b, c)] and New Mexico [Centers for Disease Control (1978a)]. Studies by Sugiyama *et al.* (1981) on baked potatoes and Lubin *et al.* (1985) on hard cooked eggs showed that *C. botulinum* would produce toxin on these substrates indicating that toxin also might be produced in salads made from them.

Canned imported marinated mushrooms were implicated in an outbreak in Canada [Centers for Disease Control (1973)] and domestic home prepared marinated mushrooms were the cause of an outbreak in Rhode Island [Centers for Disease Control (1975)]. This type of product does not receive a heat treatment sufficient to inactivate the spores of *C. botulinum* which, according to studies by Hauschild *et al.* (1975), are present in mushrooms and are capable of outgrowth and toxin production at the pH of marinated mushrooms (5.47). Studies by Sugiyama and Yang (1975) and Kautter *et al.* (1978) also indicated that fresh mushrooms could support the growth of *C. botulinum*.

Some organisms in foods can inhibit toxin production by *C. botulinum*. In sugar-containing pork for example, addition of a culture of *Enterococcus faecalis* resulted in inhibition of botulinal toxin production (Huhtanen 1986). Enterococci in ham are reportedly inhibitory to selected species of bacteria including *C. botulinum* (Riemann *et al.* 1972). *S. lactis* was shown by Saleh and Ordal (1955) to inhibit *C. botulinum* in chicken a la king, presumably due to the formation of nisin by the streptococcus. The addition of *Pediococcus cerevisiae* to semi-preserved meat products can be used to prevent the formation of botulinal toxin (Riemann and Genigeorgis 1972).

Irradiation at low doses (i.e., less than 10 kGy) has recently been approved for increasing the shelf-life of certain commodities (Code of Federal Regulations 1987). However, concern has been expressed that this process might enhance toxin production by the destruction of radiation sensitive bacteria that may act under normal conditions to prevent growth of *C. botulinum* (Anderson 1983; Teuffel 1983). The present study was initiated in order to determine whether

growth, spore formation and toxin production by *C. botulinum* types A, B and F is inhibited or enhanced when grown in metabiotic association with facultative microorganisms under controlled conditions in buffered media.

MATERIALS AND METHOD

C. botulinum Cultures

The cultures were obtained from the following sources: 17409-1(B) and 20PLALC(A) from the Centers for Disease Control, Atlanta, GA; 4(B), 383(B), 62(A), 426(A), 69(A), Langeland(F) from FDA, Washington, DC; 53(B) from U.S. Army Research and Development Command, Natick, MA; 770(B) from APHIS, Beltsville, MD; 7949(B) and 25763(A) from American Type Culture Collection, Silver Spring, MD. The spores of *C. botulinum* were prepared by growing cultures in trypticase soy broth containing 0.05% Na thioglycollate for 2 weeks at 35°C under a N_2 atmosphere. They were centrifuged, washed with water, resuspended in water, heated for 10 min at 80°C and were stored in a refrigerator at 4°C. Spore counts were made in Brewer anaerobic agar (Difco) plates incubated in an anaerobic jar (BBL) at 35°C. After incubation with the metabiotes, gram stained preparations were examined microscopically to determine the ratio of botulinal cells to total cells; phase contrast was employed to detect mature (phase bright) spores.

Metabiotic Cultures

The identified metabiotes from the USDA, Northern Regional Research Center (NRRC) were *Escherichia coli*, (B210), *Staphylococcus aureus*, (B313), *Enterococcus faecalis* (B537), *Bacillus subtilis* (B543), and *Pediococcus cerevisiae* (B1325). Other cultures were from mechanically deboned chicken homogenate (obtained from a local poultry processing plant); chicken homogenate was blended with 10 volumes distilled water and plated in plate count agar with incubation at 30°C for 2 days. Isolated colonies were picked into tryptic soy broth and incubated for 1–3 days at 30°C. Some of the cultures were identified by the API-20 system (Analytab Products, division of Sherwood Medical) as; *Serratia* sp. (6L), *Yersinia enterocolitica* (6B), *Alcaligenes* sp. (13–11) and *Pseudomonas* sp. (43–11). Thirty-two other cultures could not be readily identified but were tested for catalase production, oxygen relations and gram stained preparations were examined microscopically. These characteristics and pigment color are recorded in Table 1.

Metabiotic Culture Methods

The media used were: TS, tryptic soy broth (BBL); TST, TS with 0.05% Na thioglycollate; and BNT, a buffered medium consisting of 0.4% nutrient broth

TABLE 1.
CHARACTERISTICS OF CULTURES ISOLATED FROM
MECHANICALLY DEBONED CHICKEN MEAT

No.	Oxygen Relation	Pigment	Catalase	Gram Reaction	Morphology	No.	Oxygen Relation	Pigment	Catalase	Gram Reaction	Morphology
6B	Fac A		+	−	Short fat rods	14-10	Fac A	Yellow	+	+	Cocci in clusters
6D	Fac A	Yellow	+	−	Large cocci, pairs	14-11	Fac A		+	+	Short rods
6G	Fac A	Yellow	+	+·	Short pleo rods	14-18	Fac A		+	+	Cocci in pairs
6H	Fac A		+	+	Medium pleo rods	42-2	Fac A	Yellow	+	−	Short pleo rods
6I	Fac A		+	+	Short fat rods/cocci	42-4	Fac A	Pink	+	+	Medium pleo rods
6K	Fac A		+	−	Short rods	42-6	Fac A	Yellow	+	+	Short pleo rods
6L	Fac A		+	+	Short fat rods	42-9	Fac A	Yellow	+	−	Short fat rods
13-3	Fac A	Cream	+	+	Medium pleo rods	42-11	Fac A	Cream	+	+	Cocci in tetrads
13-4	Fac A		+	−	Short rods, pairs	43-1	Fac AN		−	+	Cocci in pairs
13-5	Fac AN		−	+	Short rods/cocci	43-2	Fac AN		−	−	Short rods
13-6	Fac AN		−	+	Short rods/cocci	43-3	Fac A		+	+	Cocci in clusters
13-8	Fac A		+	−	Short rods	43-8	Fac AN		+	−	Short fat rods
13-9	Fac A	Orange	+	+	Short pleo rods/cocci	43-9	Fac AN		+	+	Short rods
13-11	Fac A		+	−	Short rods	43-10	Fac A	Yellow	+	−	Short pleo rods
13-14	Fac AN	Yellow	+	+	Short rods/cocci	43-11	Fac AN		+	−	Short rods
14-1	Fac A	Cream	−	−	Short fat rods	43-13	Fac A	Yellow	+	+	Short pleo rods
14-4	Fac A	Cream	+	−	Medium fat rods	43-15	Fac A		+	+	Medium rods, pairs
14-7	Fac A		+	+	Medium pleo rods	43-16	Fac A		+	+	Medium pleo rods

(Difco), 1.5% TS, 0.026 M KH_2PO_4 and 0.028 M $Na_2HPO_4.7H_2O$. The pH's of these media after autoclaving were between 6.4 and 6.6. The cultures of metabiotes were grown in TS for 18 h at 30°C. They were diluted 1:1000 with sterile water and 0.1 mL was added to 10 mL medium in tubes that had been kept aerobically at ambient temperature for 5 days. The final concentration of the metabiotes was about 10^5 cells/mL; spores of *C. botulinum* were added at the same level. The cultures were incubated aerobically at 30°C for 11 days. Control cultures of *C. botulinum* alone were in BNT supplemented with 0.05% Na thioglycollate; these were incubated under N_2. The *C. botulinum* spores were also inoculated into the BNT medium without metabiotes; no growth or toxin production occurred.

Toxin Determinations

Intraperiteonal injection (0.5 mL) of 2–6 mice (15–20 g) was used to determine the presence of toxin; positive tests were those where the mice died with typical botulism symptoms. In cases where mice died without symptoms being observed, toxin was confirmed by injecting boiled extracts or by neutralization of extracts with specific antisera. Gelatin phosphate buffer (pH 6.6) was used as the diluent for quantitative toxin determinations. The $\log_{10}$ of the minimum lethal dose (MLD) was calculated from the dilution causing death of at least 50% of the injected mice.

RESULTS AND DISCUSSION

A preliminary experiment with strain 62A (data not shown) indicated that toxin was produced after 11 days incubation in the presence of the 36 cultures isolated from chicken homogenate (Table 1). The toxin concentrations were the same whether spores were added concurrently with or after two days preliminary incubation of the metabiotes. The medium was BNT which maintained the pH between 6.4 and 6.6.

The effect of other metabiotic cultures on acid production is shown in Table 2. *E. coli* in TS medium in monoculture, caused a slight drop in pH to 5.2. When *C. botulinum* was also present, the pH increased to over 6.3. A similar effect on acid development occurred with 6B. When thioglycollate was added to the medium, the pH became alkaline after incubation of *E. coli* or 6B either in single culture or when grown metabiotically with *C. botulinum*. The pH of mono-inoculated cultures of *E. faecalis* and *P. cerevisiae* was below 5.0 and was not affected by the presence of *C. botulinum* or by thioglycollate in the medium. *S. aureus* in monoculture caused a slight drop in pH to 5.6; in TS medium *C. botulinum* types A and B grown with *S. aureus* decreased the pH, but in TST medium the pH became more alkaline. *B. subtilis* grown singly

TABLE 2.
ACID DEVELOPMENT (pH) OF *C. BOTULINUM* IN METABIOTIC CULTURE

C. botulinum serotype

Metabiote[2]	Medium[3]	18 h incubation				11 days incubation			
		Control	F	B	A	Control	F	B	A
E. coli	TS	5.4	5.4	5.4	5.4	5.2	7.6	6.3	6.4
	TST	5.4	5.4	5.4	5.5	7.4	7.2	7.3	7.8
E. faecalis	TS	5.0	5.0	5.0	5.0	4.6	4.6	4.6	4.6
	TST	4.8	4.8	4.8	4.8	4.6	4.5	4.6	4.5
P. cerevisiae	TS	6.2	6.0	6.4	5.9	4.5	4.5	4.5	4.5
	TST	6.2	6.0	6.1	6.1	4.5	4.4	4.4	4.4
S. aureus	TS	6.5	6.6	6.7	6.6	5.6	5.9	5.0	5.0
	TST	5.8	6.0	6.0	5.9	5.6	6.2	6.6	6.0
B. subtilis	TS	7.0	7.2	7.0	7.1	8.3	8.1	7.0	7.1
	TST	7.0	7.0	7.0	7.0	8.4	8.3	8.0	8.4
6L[4] (Serratia sp.)	TS	7.2	7.2	7.2	7.2	8.4	8.2	7.9	8.2
	TST	7.2	7.2	7.2	7.0	8.5	8.2	8.0	8.2
6B (Y. enterocolitica)	TS	5.8	5.8	5.8	5.8	5.2	5.7	5.6	6.4
	TST	6.2	6.2	6.2	6.2	8.4	8.4	6.9	8.3

[1]Serotypes were Langeland (F), 17409-1(B) and 62(A). The spore concentrations were, respectively; 3.7, 3.8 and 1.8 × 10⁵/mL. The spores and metabiotes were co-inoculated.
[2]Metabiotes were added at levels of about 1×10^5/mL.
[3]TS medium was trypticase soy broth (BBL) which contains 0.25% glucose; TST medium was TS supplemented with 0.05% Na thioglycollate. Cultures were incubated aerobically at 30°C.
[4]Cultures 6L and 6B were isolated from mechanically deboned chicken meat.

produced an alkaline reaction after 11 days incubation, but with *C. botulinum* A or B in TS medium the pH decreased.

Table 3 shows toxin production by these cultures. Very little toxin was produced during the 18 h incubation period and then only in the TS medium by Langeland F grown with 6L, and by 62A grown in the presence of 6L and *B. subtilis*. The level of toxin was log MLD 0.78, which corresponded to a dilution of 1 part culture to 2 parts of the gelatin phosphate buffer.

After 11 days incubation, toxin formation by Langeland F was greatest when it was grown in the presence of *S. aureus* or 6B; toxin was undetectable when it was grown with *E. faecalis* or with *P. cerevisiae,* these metabiotes also produced the lowest pH's (Table 2). A very low toxin level resulted from the metabiotic growth of the type F strain with 6L and no toxin was found when it

TABLE 3.
TOXIN PRODUCTION[1] BY *C. BOTULINUM* IN METABIOTIC CULTURE

Metabiote	Medium[2]	C. botulinum serotype[2]							
		18 h incubation				11 days incubation			
		Control	F	B	A	Control	F	B	A
E. coli	TS	0	0	0	0	0	1.78	0	5.78
	TST	0	0	0	0	0	1.78	1.78	3.60
E. faecalis	TS	0	0	0	0	0	0	0	0
	TST	0	0	0	0	0	0	0	0
P. cerevisiae	TS	0	0	0	0	0	0	0	0
	TST	0	0	0	0	0	0	0	0
S. aureus	TS	0	0	0	0	0	4.60	1.60	5.78
	TST	0	0	0	0	0	2.60	2.60	4.78
B. subtilis	TS	0	0	0	0.78	0	0	2.78	4.78
	TST	0	0	0	0	0	1.78	1.78	2.78
6L (Serratia sp.)	TS	0	0.78	0	0.78	0	0.78	2.90	2.78
	TST	0	0	0	0	0	0.78	2.60	2.78
6B (Y. enterocolitica)	TS	0	0	0	0	0	2.90	0	5.78
	TST	0	0	0	0	0	2.78	2.90	1.78

[1]Mice (2–6 per dilution) were injected i.p. with 0.5 mL of centrifuged culture supernate. Positive response was death with typical botulinal symptoms. A toxin level of 0 meant no deaths at 1:2 dilution, which was the lowest level tested. Toxin levels are given as log MLD/mL. *C. botulinum* in mono culture did not show growth or toxin production.
[2]See Table 1.

was grown with *B. subtilis* in TS medium. These low toxin levels may have been due to toxin destruction by the alkalinity produced during the incubation; these cultures had pH's of 8.4 or 8.5.

Toxin formation by type B was also inhibited by *E. faecalis* and *P. cerevisiae* in both TS and TST-media. No detectable toxin was produced when type B was grown metabiotically with *E. coli* or with 6B.

E. faecalis and *P. cerevisiae* inhibited toxin formation by 62A as well. Detectable levels of toxin were formed in the presence of the other metabiotes, although there was a decrease in toxin levels in cultures with pH's over 8.0. The highest toxin level was with *S. aureus* even though the pH of this culture was 5.0, a level considered to be too low for growth of *C. botulinum*. It is possible that the acidity developed after toxin was formed.

The results of a more comprehensive study in the buffered medium (BNT) are shown in Table 4. As in the previous studies, mono-cultures of *C. botulinum* in these media did not grow and no toxin was detectable in the mouse test. Toxin

METABIOTIC TOXIN PRODUCTION[1] BY 10 STRAINS OF *C. BOTULINUM*

C. botulinum culture[2]

Metabiote	Type A					Type B				
	a	b	c	d	e	f	g	h	i	j
E. coli	3.38	3.08	2.78	1.78	3.78	1.78	2.78	1.78	0.78	3.38
S. aureus	3.08	4.28	3.98	4.28	4.28	2.78	3.68	3.68	0.78	3.38
43-11 (Pseudomonas sp.)	0.78	1.48	2.78	1.78	3.08	1.78	1.78	1.78	0	1.78
P. cerevisiae	0.78	4.28	0	3.98	3.38	1.78	3.38	3.38	0.78	1.78
6L (Serratia sp.)	2.78	1.78	3.38	3.08	2.78	2.78	2.78	1.78	0.48	2.78
13-11 (Alcaligenes sp.)	3.08	3.38	1.78	1.78	3.38	2.78	2.78	2.78	0.48	2.78
None-CM[3]	3.38	4.28	4.58	4.58	4.58	3.38	4.58	4.58	0.48	3.38
" BNT	3.38	3.98	4.58	3.98	4.58	3.38	4.58	4.58	0.48	3.38
" BNTT	3.38	4.28	4.58	3.98	4.58	3.08	ND[4]	4.28	0.48	3.38

[1]Cultures were co-inoculated and were incubated 11 days at 30°C.
[2]Type A cultures were: a-69, b-ATCC 25763, c-426, d-20PLALC, e-62; type B cultures were f-4, g-53, h-770, i-383, j-ATCC 7949.
[3]The mono-inoculated cultures of *C. botulinum* were grown in the following media: cooked meat (CM); buffered nutrient:trypticase soy broth (BNT); BNT with 0.05% Na thioglycollate (BNTT). These were incubated under N_2.
[4]ND—not done.

formation by 69A was decreased by culture 43-11, tentatively identified as *Pseudomonas sp.*, and by *P. cerevisiae*. The log MLD was 3.38 for mono-inoculated *C. botulinum* grown in cooked meat or BNT media under anaerobic conditions; this decreased to log MLD 0.78 when grown with these metabiotes. There was little or no decrease in toxin production when 69A was grown with the other metabiotes. ATCC 25763 produced less toxin with the metabiotes 43-11 and 6L. Strain 426A produced less toxin in the presence of all metabiotes tested but the reduction was greatest with *P. cerevisiae* where no detectable toxin was found. Some toxin was produced with metabiote 13-11 (log MLD 1.78); but maximum toxin (log MLD 3.98 from the BNT controls) was not attained except when grown with *S. aureus*.

Toxin production by 20PLALC type A also appeared to be inhibited by 43-11 and 13-11 as well as by *E. coli*. Less toxin was produced by strain 62A in

the presence of 6L and 43-11 although the lowest concentration was log MLD 2.78. The only metabiote completely inhibiting toxin production by any of the type A cultures was *P. cerevisiae* when grown with strain 426. Of the metabiotes tested *S. aureus* produced conditions most conducive to toxin formation by type A strains.

Strain 4B produced less toxin when grown in the presence of the metabiotes; the smallest amounts were produced with the metabiotes *E. coli*, 43-11 and *P. cerevisiae*. Strain 53B also produced lesser amounts of toxin with 43-11 while strain 770B produced less with *E. coli*, 43-11 and 6L. Strain 383B produced very little toxin with the highest concentrations being log MLD 0.78. No detectable toxin was produced by this strain in the presence of 43-11. Toxin formation by ATCC strain 7949 was inhibited by the metabiotes 43-11 and *P. cerevisiae* although a small amount of toxin was produced.

The metabiotic cultures were examined microscopically for growth and sporulation of *C. botulinum*. Table 5 shows the ratios of *C. botulinum* cells to total cells in each field (the botulinal cells were clearly distinguishable as paired rods). There was no apparent correlation of cell numbers with toxin production. Cells of culture 69A for example, constituted 90% of the 11 day population when

TABLE 5.
METABIOTIC GROWTH OF AND SPORE PRODUCTION BY *C. BOTULINUM* TYPE A[1]

Meabiote	Culture[2]				
	a	b	c	d	e
E. coli	50(2)	10(80)	90(80)	90(10)	90
S. aureus	5	1	2(0.1)	10	1(1)
43-11	5(20)	1	1(100)	0	0
P. cerevisiae	90	2(80)	0	1	2(10)
6L	10	1(100)	5(20)	10	2
13-11	20(10)	2(80)	2(50)	5	5
None CM[2]	(50)	(0)	(90)	(1)	(90)
None BNT	(50)	(10)	(10)	(0)	(10)
None BNTT	(0)	(90)	(0)	(0)	(1)

[1]Medium was BNT incubated at 30°C for 11 days. Figures represent *C. botulinum* cells as the percentage of total cells determined microscopically from gram stains. Figures in parentheses represent spores (phase bright) as the percentage of total *C. botulinum* cells.
[2]See Table 4 footnote.

grown with the *P. cerevisiae* metabiote but the toxin level was only log MLD 0.78; while with *S. aureus* it made up 5% of the population and the toxin level was log MLD 3.08. Spore formation by this strain did not appear to correlate with toxin formation; the highest level of toxin was formed in BNTT medium but no spores were present at 11 days. On the other hand 20% of the *C. botulinum* cells contained spores when grown with the 43-11 metabiote but the toxin level was only log MLD 0.78. The lack of spore:toxin correlation was also apparent with culture 20PLALC; it produced only a few spores; but toxin levels were as high as log MLD 4.58. The type B cultures (Table 6) similarly showed no correlation of toxin to spore numbers. *S. aureus* appeared to inhibit spore formation by both the type A and type B strains of *C. botulinum* while metabiotic growth with *E. coli* or 13-11 produced the greatest numbers of spores.

These studies indicate that *C. botulinum* growth and toxin production is possible in aerobically incubated media by the concomitant growth of facultatively aerobic or anaerobic microorganisms. Although there is some diminution of toxin formation when grown metabiotically with some bacteria, total inhibition occurs only as a result of acid production in sugar-containing unbuffered media. The results indicate that *C. botulinum* competes successfully with a wide variety of microorganisms for available nutrients even when it is inoculated into mature

TABLE 6.
METABIOTIC GROWTH OF AND SPORE PRODUCTION BY *C. BOTULINUM* TYPE B[1]

Metabiote	f	g	h	i	j
			Culture		
E. coli	20	20(20)	10(10)	20(10)	20(10)
S. aureus	1	1	0	1	2(1)
43-11	2	2(90)	1	1	2(10)
P. cerevisiae	2	1(10)	1	5	50(10)
6L	2	1(10)	10(10)	1(10)	10(10)
13-11	10(10)	10(50)	10(10)	10	2(10)
None CM	(100)	(95)	(50)	(0)	(50)
None BNT	(0)	(0)	(0)	(1)	(0)
None BNTT	(0)	(0)	(95)	(0)	(20)

[1]See Table 5 footnotes.

cultures of metabiotes as in the case where 62A spores were added to 2 day cultures of bacteria isolated from chicken meat.

These results substantiate the results of early workers who indicated that *C. botulinum* produced toxin in the presence of several aerobic or facultative microorganisms. These workers, however, used organisms of uncertain serotype, for example Shippen (1919) described his organism as *Bacillus botulinus*. PH was not measured and toxin was determined in various animals such as chickens, horses, frogs and cats. Quortrup and Sudheimer (1943) used type C *C. botulinum* and used the mouse assay for toxin. They concluded that type C toxin formed in marshes as the result of concomitant growth of oxygen-consuming bacteria. The results of the present study indicate that enhancement of toxin production occurs in the presence of a variety of facultatively aerobic or anaerobic bacteria, but some bacteria can also inhibit toxin formation if enough fermentable carbohydrate is present. The concept of limited "lebensraum" in growing cultures does not appear to apply to the metabiotic association of *C. botulinum* with the bacteria studied here; inoculation of *C. botulinum* spores into fully grown bacterial cultures did not prevent toxin formation.

The effect of elimination of associated microorganisms on the subsequent growth of *C. botulinum* (whether by heat, irradiation or other means) was not studied here but several scenarios may occur. In an aerobically incubated foodstuff treated to decrease the numbers of nontoxin producing microorganisms, *C. botulinum* growth and toxin production would be prevented or slowed, but in anaerobically incubated, carbohydrate-containing food, similarly treated, toxin production might be enhanced if acid-producing bacteria were destroyed by the treatment. This was observed in irradiated bacon that contained sugar and a streptococcus culture (Huhtanen 1986).

It is interesting that *C. botulinum* was able to grow and produce toxin in buffered media when grown concurrently with a variety of microorganisms, most of which reduce oxidation reduction potentials by using atmospheric oxygen as the terminal electron acceptor, but it is particularly noteworthy that growth and toxin formation took place when it was grown with bacteria incapable of utilizing atmospheric oxygen (*P. cerevisiae*).

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